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# Imaging neuron-glia interactions in the enteric nervous system

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## Abstract

The enteric nervous system (ENS) is a network of neurons and glia within the wall of the gastrointestinal tract that is able to control many aspects of digestive function independently from the central nervous system. Enteric glial cells share several features with astrocytes and are closely associated with enteric neurons and their processes both within enteric ganglia, and along interconnecting fiber bundles. Similar to other parts of the nervous system, there is communication between enteric neurons and glia; enteric glial cells can detect neuronal activity and have the machinery to intermediate neurotransmission. However, due to the close contact between these two cell types and the particular characteristics of the gut wall, the recording of enteric glial cell activity in live imaging experiments, especially in the context of their interaction with neurons, is not straightforward. Most studies have used calcium imaging approaches to examine enteric glial cell activity but in many cases, it is difficult to distinguish whether observed transients arise from glial cells, or neuronal processes or varicosities in their vicinity. In this technical report, we describe a number of approaches to unravel the complex neuron-glia crosstalk in the ENS, focusing on the challenges and possibilities of live microscopic imaging in both animal models and human tissue samples.

## 1 Introduction

2  
3 Many aspects of gastrointestinal function are controlled without major inputs from the brain.  
4 Instead, the enteric nervous system (ENS), a ganglionated neuronal network that resides  
5 within the gut wall, autonomously controls gastrointestinal motility, secretion, and blood flow  
6 (Furness, 2000). The ENS develops from neural crest cells that migrate and proliferate  
7 extensively to eventually form a network of interconnected ganglia throughout the entire  
8 length of the gut (Sasselli et al., 2012; Obermayr et al., 2013). Similar to other parts of the  
9 nervous system, the ENS comprises both a neuronal and a glial component. Enteric glial cells  
10 are located in close contact with enteric neurons within the ganglia, along interganglionic  
11 connectives of the myenteric and submucosal plexus, and can also be found in the  
12 extraganglionic layers of the gut wall (Gershon and Rothman, 1991). In contrast to other parts  
13 of the peripheral nervous system, the ENS is quite exceptional: it lacks coats of connective  
14 tissue that surround nerve cell bodies and fibers and is therefore more reminiscent to the  
15 central nervous system (Cook and Burnstock, 1976; Gabella, 1981). Enteric glial cells share  
16 many phenotypical features with astrocytes, and were for a long time also believed to function  
17 mainly as support cells for neurons. However, in the last two decades this dogma has  
18 gradually been abandoned and considerable progress has been made in understanding enteric  
19 glial function, most of which has been covered in recent reviews (De Giorgio et al.,  
20 2012; Gulbransen and Sharkey, 2012; Neunlist et al., 2013).

21  
22 Although the concept of “tripartite synapses”, as introduced in the central nervous system,  
23 (Perea et al., 2009) is far from established in the ENS, enteric glial cells are also considered to  
24 be active partners in neurotransmission (Ruhl et al., 2004). They display dynamic responses to  
25 neuronal inputs and sequester and release neuro-active factors that could modulate  
26 neighboring pre- and postsynaptic elements. However, the detection of enteric glial activity,  
27 especially when their interaction with enteric neurons is targeted, is not unambiguous. The  
28 close proximity between enteric glia and neurons and their processes makes optical  
29 discrimination between signals originating from specific cells particularly challenging.

30  
31 In this technical report, we describe a number of approaches to disentangle the complex  
32 neuron-glia crosstalk in the ENS, in both animal models and human tissue. We discuss the  
33 techniques that have been used to examine neuron-glia interactions in the animal and human  
34 ENS with an emphasis on live intracellular calcium concentration ( $[Ca^{2+}]_i$ ) imaging and  
35 present some novel analysis tools that serve this purpose.

## 38 Neurons and glia: close neighbors in the gut

39  
40 Enteric glial cells resemble astrocytes in several ways, including the expression of the  
41 intermediate filament glial fibrillary acidic protein (GFAP) (Jessen and Mirsky, 1980) and the  
42  $Ca^{2+}$  binding protein, S100 $\beta$  (Ferri et al., 1982). The intimate association between glia and  
43 neurons within enteric ganglia has been revealed by co-immunolabeling of gut tissue with  
44 GFAP or S100 $\beta$  with neuronal markers, such as HuC/D (**Figure 1A, B**). In addition, the  
45 transcription factor SRY box-containing gene 10 (Sox10), which is expressed by multipotent  
46 ENS precursors (Paratore et al., 2002; Bondurand et al., 2003), is also expressed by mature  
47 enteric glia (Young et al., 2003), and is ideally suited for quantification purposes because of  
48 its selective nuclear localization (**Figure 1C**) (Hoff et al., 2008). Enteric glial cells closely  
49 embrace nerve fibers and varicose release sites both within enteric ganglia and along the  
50 interconnective fiber tracts (**Figure 1D-F**) (Hanani and Reichenbach, 1994; Vanden Berghe

and Klingauf, 2007). As a consequence, the optical segregation of signals arising from either neurons or glia in live imaging experiments is not straightforward.

Microscopic imaging techniques have provided invaluable information about several aspects of neuronal signaling in both the developing and adult ENS (Schemann et al., 2002; Vanden Berghe et al., 2008; Hao et al., 2012). In particular, chemo- and mechanosensitivity of various classes of enteric neurons has been uncovered using both voltage-sensitive and  $\text{Ca}^{2+}$  indicator dyes (Smith et al., 2007; Schemann and Mazzuoli, 2010). Strong evidence for communication between enteric neurons and glia comes from a series of studies using live imaging in both *ex vivo* wholemount preparations of gut and in cell cultures of the ENS (Gomes et al., 2009; Gulbransen and Sharkey, 2009; Gulbransen et al., 2010). In addition, in a study by Broadhead et al., interaction between neuronal and glial cells was shown following spontaneous or induced physiological activity (Broadhead et al., 2012). In all studies, purinergic signaling pathways have been identified as the primary mechanism of transmission.

### **Live imaging of neuron-glia interactions: *in vitro* versus *ex vivo***

Optical information generated by multiple sources is always convoluted due to the diffraction limitations of optical microscopy. To address this, we estimated the contribution of any given signal in the pixels directly neighboring a structure of interest as recorded by a widefield fluorescence microscope equipped with a CCD camera. Primary ENS cultures (including both neurons and glia) were loaded with the  $\text{Ca}^{2+}$  indicator dye, Fluo4-AM, and stimulated by 75mM  $\text{K}^{+}$  depolarization (**Figure 2**). To measure the change in fluorescence, a region of interest (ROI) was drawn over a neuronal bouton. We found that even with limited optical resolution (widefield, 20x, NA = 0.75, pixel width: 623 nm), the signal contribution drops sharply outside of any structure that can be picked out intuitively by an observer (**Figure 2C**). Simple rectangular regions of interest (ROIs) are sufficient to calculate the cellular signals and polygon-shaped ROIs do improve signal to noise ratios due to inclusion of larger cell areas (**Figure 2D-F**). Thus, at least in *in vitro* experiments, careful drawing of ROIs at least 1  $\mu\text{m}$  away from each other may be sufficient to separate signals coming from structures situated in each other's vicinity.

The ENS, due to its planar organization in *ex vivo* preparations, is very attractive to investigate with imaging techniques. However the assumption of 2D structure is only valid when entire cells are considered. Once synaptic contacts and cellular processes are of interest, the analysis faces all the technical problems that are associated with 3D organization and ROIs will easily incorporate scattered light emanating from structures close by. Hence, the intimate relationship between enteric glia, neurons and their processes (**Figure 1**) entails the risk that signals arising from enteric varicosities and fibers are interpreted as being of glial origin. This is an important confounding factor that may cause the false impression that glial cells respond as fast to electrical and depolarizing stimuli as do neurons. Therefore, to make use of the fundamental physiological difference between neurons and electrically passive non-excitable glia (Hanani et al., 2000; Gulbransen and Sharkey, 2012) we suggest, in combination with using lenses with sharp focal depths, to consistently apply known stimuli (e.g. electrical stimulation) to identify neuronal structures, which then serve as a guide to draw regions at least 1  $\mu\text{m}$  away from other structures in order to minimize false interpretation.

1 The clear delineation of the cells and compartments of interest in *ex vivo* gut preparations is  
2 further complicated by the fact that these ganglia are on a contractile muscle layer, which  
3 even with pharmacological inhibition and mechanical restraining can still cause movement  
4 artifacts. This further complicates accurate analysis, especially of smaller structures and cell  
5 compartments (e.g. varicosities, parts of glial processes). To correct for residual movements,  
6 we use translation stabilization routines (Bisschops et al., 2006;Gallego et al., 2008), which  
7 recently have been expanded to also correct for more complex movements like rotation and  
8 torque.

## 11 **Differentiating between neuronal and glial responses: response timing and shape**

13 Although originally not intended for studying neuron-glia interactions, data from pioneering  
14 studies showing that several neuroligands can elicit  $\text{Ca}^{2+}$  transients in cultured enteric glial  
15 cells have already indicated the potential for glial participation in enteric neurotransmission  
16 (Kimball and Mulholland, 1996;Zhang et al., 1997;Zhang et al., 1998;Garrido et al., 2002). In  
17 an alternative approach to directly measure the sensitivity of enteric glial cells to neuronal  
18 transmitters, we used an immortalized rat enteric glia cell line (CRL-2690) (Ruhl et al., 2001)  
19 and confirmed that neurotransmitters known to elicit fast excitatory potentials in enteric  
20 neurons can directly induce  $[\text{Ca}^{2+}]_i$  changes in enteric glial cells (Boesmans et al., 2013). In  
21 contrast, enteric glia did not respond to high  $\text{K}^+$  depolarization. The absence of neurons in  
22 these cultures obviously eliminates the problems illustrated above, but also excludes the  
23 possibility that these cells are activated secondary to neuronal activation. However, in mixed  
24 cultures of neurons and glia this is not the case (Gomes et al., 2009). By specifically  
25 stimulating enteric neurons while monitoring the secondary glial responses, an adenosine  
26 triphosphate (ATP)-dependent paracrine communication pathway between enteric neurons  
27 and glia was revealed.

29 This typical ATP sensitivity was also found in enteric neuron-glia co-cultures obtained from  
30 adult mouse gut where the neuronal and glial  $\text{Ca}^{2+}$  fingerprint was used to identify specific  
31 cell types (Laranjeira et al., 2011). Indeed, enteric neurons display a strong and fast  $\text{Ca}^{2+}$   
32 response to high  $\text{K}^+$  depolarization, electrical field stimulation (EFS) and the nicotinic agonist  
33 dimethylphenylpiperazinium (DMPP). Enteric glial cells, on the other hand, do not respond to  
34 these stimuli directly, but show delayed responses that can be modulated by intervening with  
35 several components of purinergic signaling (Gomes et al., 2009;Laranjeira et al., 2011)  
36 (**Figure 3**). Due to these timing differences, it is possible to construct “activity-over-time”  
37 (AoT, **Figure 3D**) images that identify cells which exhibit a change in fluorescence intensity  
38 above baseline noise. These images appear similar to immunostainings, but a physiological  
39 response is represented instead of the structural information (custom developed algorithm in  
40 Igor Pro, Wavemetrics).

42 To further characterize the timing of the responses, we developed a routine in which the  
43 shortest distance to an active neuronal fiber was computed and transformed in a color coded  
44 image (**Figure 3F-J**). The computation of distance can be performed either on an  
45 immunohistochemical staining or on one of the AoT images generated from live recordings. In this  
46 way we are able to test whether secondary responses, for instance responses in glial cells,  
47 emerge earlier if they are physically closer to active neuronal fibers. We found that the timing  
48 of glial responses does correlate with spatial aspects since cells closer to a neuronal  
49 component have a higher likelihood of responding to a neuronal stimulus (**Figure 3I**), thus

1 further corroborating the fact that it is a diffusible factor that mediates the communication  
2 from enteric neurons to glia.

3  
4 Taking all these technical issues into consideration, it is possible to isolate the net responses  
5 from neurons and glial cells in complex tissues. This reveals that the  $\text{Ca}^{2+}$  transients in both  
6 cell types have typical shapes and kinetics to stimuli like EFS, high  $\text{K}^{+}$  depolarization and fast  
7 neurotransmitters: in neurons, fast and linear upstrokes reaching their maximum in a couple of  
8 seconds are followed by a (bi)-exponential decay, while in glia a secondary close-to-Gaussian  
9 shaped response is observed. This typical fingerprint can thus be used also in tissue to identify  
10 different cell types (**Figure 4**).

11  
12 It is of note that enteric glial cells display higher baseline fluorescence after Fluo4 loading  
13 compared to neurons (arbitrary fluorescence units, neurons: 260.7 vs glia: 339.6,  $p < 0.05$ ,  
14  $n=103$ , data from 3 animals, unpaired t-test), a difference that is more pronounced in *ex-vivo*  
15 tissue preparations in comparison to primary culture. Using the ratiometric dye Fura2, we  
16 tested whether this difference was due to higher resting level of intracellular  $\text{Ca}^{2+}$ . We found  
17 the differences (340/380 ratio, neurons: 0.3442 vs glia: 0.3623;  $p < 0.05$ ,  $n=120$ , data from 3  
18 animals, unpaired t-test) to be only very small and definitely not sufficient to explain the large  
19 differences in resting Fluo4 fluorescence. This was further confirmed by using Rhod2, a non-  
20 ratiometric  $\text{Ca}^{2+}$  indicator with an even higher  $K_d$ , which is classically used in astrocyte  
21 research (Mulligan and MacVicar, 2004; Takano et al., 2006) and can also be used to load  
22 enteric glial cells (Gulbransen and Sharkey, 2009). Again, higher resting levels were observed  
23 in glial cells compared to neurons (arbitrary fluorescence units, neurons: 330.8 vs glia: 419.4,  
24  $p < 0.05$ ,  $n=113$ , data from 3 animals, unpaired t-test). Taken together, this suggests that glial  
25 cells take up and/or metabolize the AM ester more easily, which is probably a reflection of a  
26 higher metabolism.

## 27 28 29 **Novel genetic tools for studying enteric neuron-glia interactions**

30  
31 Apart from identification during live recordings and *post-hoc* analysis, many new genetic  
32 tools are available to label specific cells. Given the analogy between enteric glia and  
33 astrocytes, transgenic animals in which reporter proteins, such as green fluorescent protein  
34 (GFP) derivatives, have been placed under the direct control of GFAP or S100 $\beta$  regulatory  
35 elements to study astrocytic function can also be used to visualize enteric glial cells in live  
36 imaging experiments. Furthermore, the conditional expression of fluorescent reporters by Cre-  
37 Lox recombination technology enables identification of enteric glia as illustrated by Joseph et  
38 al., (Joseph et al., 2011), who combined GFAP-Cre (Zhuo et al., 2001) and GFAP-CreER<sup>T2</sup>  
39 (Hirrlinger et al., 2006) mice with Rosa26ReYFP reporter mice (Srinivas et al., 2001) for  
40 lineage tracing purposes. Time-dependent induction of Cre in the Sox10-iCreER<sup>T2</sup> transgenic  
41 mouse line generated by Laranjeira et al., not only allows fate mapping of multilineage ENS  
42 precursors and labeling of enteric neurons (Sasselli et al., 2013), but also elegantly enables  
43 marking enteric glial cells only (Laranjeira et al., 2011). A big advantage of such a genetic  
44 system is the fact that individual cells can be labeled, thus allowing appreciation of the  
45 cellular morphology as opposed to immunostaining of adjoining cells. These transgenic  
46 mouse lines can aid in the examination of enteric neuron-glia interactions in several ways.

47  
48 Fluorescent reporter lines are favorable over *post-hoc* immunohistochemistry for the  
49 identification and localization of glia in live imaging experiments since imaging can be  
50 performed directly in the cells of interest. It is for these reasons that S100 $\beta$ -eGFP mice have

(Vives et al., 2003) been used in  $\text{Ca}^{2+}$  and nitric oxide (NO) imaging studies (Gulbransen and Sharkey, 2009; Lavoie et al., 2011; MacEachern et al., 2011). Also, ENS cultures have been generated from Sox10-iCreER<sup>T2</sup>:R26R<sup>FP635</sup> mice to characterize the  $\text{Ca}^{2+}$  responses of newborn neurons and genuine enteric glial cells upon a number of stimuli (Laranjeira et al., 2011). Here, the conditional expression of the red fluorescent protein FP635 (Shcherbo et al., 2009) was used to identify neurons that were derived from cultured enteric glial cells.

Another application of transgenic methods lies in the recent development of several optogenetic tools, an opportunity that has yet to be exploited in ENS research. The core instruments of these novel techniques are genetically-encoded optical indicators (Knopfel, 2012) and actuators (Fenno et al., 2011) that enable interrogation and manipulation of cell-to-cell interactions with cellular to subcellular resolution (Miesenbock, 2009). Among the optogenetic reporter molecules, genetically-encoded  $\text{Ca}^{2+}$  indicators (GECIs), such as GCaMPs, allow imaging of  $\text{Ca}^{2+}$  signaling in genetically defined cell populations, thus providing a powerful means to study neuron-glia interactions in the ENS. Recently, a reporter mouse was developed that expresses GCaMP3 (Tian et al., 2009) in a Cre-dependent manner in the *Rosa26* locus (Zariwala et al., 2012). By using the *Wnt1-Cre* transgene (Danielian et al., 1998) to conditionally express GCaMP3 in neural crest derivatives, we found that this system can also be used to perform  $\text{Ca}^{2+}$  imaging in enteric neurons and glia (**Figure 5**). Because tissue loading steps are omitted, tissue viability can be increased and background signals (e.g. from the underlying smooth muscle layers) reduced. However, to fully employ the advantages of these genetically-encoded indicators, they should ideally be expressed in enteric neurons or glial cells specifically. This will help to overcome the earlier illustrated problems caused by the close proximity between enteric neurons and glia. In addition, depending on the specifics of the transgenic method used, they can potentially enable monitoring of events in cellular subtypes and/or subcellular compartments.

## Imaging neuron-glia crosstalk in the human enteric nervous system

Because most investigations have been carried out using *in vitro* and *ex vivo* animal models, and given the difficulty to obtain healthy human gut tissues for experimental purposes, our current knowledge about enteric neuron–glia interactions in the human gut is rather poor. The limited information about human enteric glia function originates from *in vitro* studies using enteric glia isolated from surgical resection specimens. These studies indicated that human enteric glia actively participate in inflammatory responses (Cirillo et al., 2011) and host-bacteria crosstalk (Turco et al., 2013). However, even though these glial cells were obtained from ‘macroscopically normal’ tissues, there is still the possibility that measurements were influenced by the fact that the resection specimens were collected from patients suffering from a variety of severe diseases.

For these reasons we have recently developed a method to culture human enteric glial cells isolated from routine intestinal biopsies (Boesmans et al., 2013). After careful removal of the mucosa, the submucosal plexus is enzymatically digested following previously described procedures (Cirillo et al., 2011), ganglia are selected and cells cultured on glass coverslips to perform live imaging studies. By implementing this technique we found that similar to rat enteric glia, also human enteric glial cells can be activated by neurotransmitters known to elicit fast excitatory responses in the ENS (Boesmans et al., 2013).

1 Of course, in order to fully understand neuron-glia interactions in the human gut, also these  
2 should ideally be studied in intact *ex vivo* preparations. With the recently developed optical  
3 imaging approach (Cirillo et al., 2012), we have shown that it is feasible to record neuronal  
4 activity by means of Fluo4  $\text{Ca}^{2+}$  imaging in submucosal ganglia dissected from duodenal  
5 biopsies obtained from humans. This technique also allows exploring human enteric glia  
6 function (**Figure 6**). Again, analysis is not straightforward, even in comparison to the ENS of  
7 animal models, as the cells in the human enteric ganglia are organized in a far more three  
8 dimensional manner than in small animals. Nerve bundles and glial projections together form  
9 a complex structure (the ganglionic capsule) that surrounds neurons and glial cells (**Figure**  
10 **6A, B**). Moreover, the presence of fasciculated bundles interconnecting adjacent ganglia adds  
11 to the intricacy of optical recordings from such ganglia. This makes correct interpretation of  
12 glial activation and discrimination between neuronal and glial signaling difficult, but not  
13 impossible. By analogy with the animal tissue experiments, it is still feasible, with careful  
14 attention to focus, movement, and analysis issues, to distinguish between signals originating  
15 from glia and neurons (**Figure 6C-E**). Here again, glial signals are delayed with respect to the  
16 responses observed in neuronal compartments.

## 19 **Conclusions and perspectives**

21 Despite significant progress in understanding enteric glia function, the exact signaling  
22 mechanisms and possible ‘gliotransmitters’ that act in a physiological context such as during  
23 the different gastrointestinal motility patterns remain elusive. Nonetheless, several studies  
24 indicate that enteric glial cells are active partners in enteric neurotransmission. In particular,  
25 the aforementioned live imaging studies have provided invaluable information about how  
26 enteric glial cells can detect neuronal activity (Gomes et al., 2009; Gulbransen and Sharkey,  
27 2009; Gulbransen et al., 2010; Broadhead et al., 2012; Gulbransen et al., 2012). As illustrated,  
28 the intimate association between enteric glia and neurons warrants careful experimental and  
29 analytic considerations. Enhancing optical resolution by using confocal recordings would  
30 obviously help towards avoiding false interpretation of the signal’s origin especially when  
31 two structures truly overlap. However, this should be combined with deconvolution methods  
32 as resolution along the optical axis is never as high as in the XY plane. Confocal recordings  
33 can definitely improve the spatial resolution, but as we have shown, also timing is important  
34 to tell different responses apart, an advantage that is lost in classical point scanning confocals  
35 due to limitations in scan speed. Therefore, spinning disk confocal recordings may offer an  
36 intermediate solution. The toolbox of optical, off-line analysis and genetic approaches that we  
37 present here, can aid in disentangling the complex interplay between neurons and glia, thereby  
38 producing experimental access to bridge differing conclusions.

## **Conflict of interest statement**

The authors have no financial or other conflicts to disclose.

## **Author contributions**

Concept and design, interpretation of data, statistical analysis, drafting and editing of the manuscript were done by W.B. and P.V.B; W.B., M.A.M., N.W. and M.M.H. performed experiments, image analysis and contributed to paper writing; C.C. performed and analyzed experiments on human enteric glia and was involved in paper writing; J.T. was responsible for mucosal biopsy collection; P.V.B. and M.A.M. wrote analysis software; and J.T. and P.V.B. obtained funding.

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## Figure Legends

**Figure 1. The relation between enteric neurons and glia visualized by immunohistochemistry.** (A-C) Maximum projection of colonic myenteric ganglia of mice stained for the pan-neuronal marker HuCD (red) and enteric glial cell markers (green) S100 $\beta$  (A), GFAP (B) and Sox10 (C). (D-E) Maximum projection composed of a stack of 2 confocal images of a mouse colonic myenteric ganglion labeled with antibodies for S100 $\beta$  (glia, yellow) and HuCD (neurons, magenta) and for vAChT (cyan) and substance P (grays) revealing the close apposition of neuronal fibers and varicosities with enteric glial cells. Scale bars: 25  $\mu$ m (A-C), 10  $\mu$ m (D).

**Figure 2. Quantification of Fluo4 signals in regions of interest (ROI).** (A-C) Example of a neuronal fiber and bouton at rest (A) and depolarized with 75 mM K<sup>+</sup> (B). In (C) the amplitude (at rest in gray, depolarized in red circles) along the line marked green in A and B is plotted. As seen, especially in the inset, the signal drops sharply outside the intuitively drawn ROI (gray shade bar in C and gray box in B): the last pixel included contains 7.5 % of the signal's maximal amplitude, while the first pixel outside the ROI only contains 2.5%. (D-F) Analysis of a situation in which a neuronal fiber crosses a glial cell. The cyan region of interest includes a mixture of both neuronal (purple) and glial (red, blue, green) information. Smaller regions of interest (rectangular or polygon shaped) away from the nerve fiber identify pure glial signals with improving signal to noise ratios (F) for larger areas included. Scale bars: 10  $\mu$ m (A, B), 20  $\mu$ m (D).

**Figure 3. Ca<sup>2+</sup> imaging of enteric neuron-glia interactions in primary enteric nervous system cultures.** (A-B) Gray scale images of a patch of cultured mouse enteric neurons and glia loaded with Fluo4 at rest (A) and depolarized by 75 mM K<sup>+</sup> application (5 sec, B). Note the large increase in fluorescence of the group of neuronal cell bodies in the center of the image. (C) Magnification of the frame indicated in (B) showing in detail the neuronal fibers and varicosities as depolarized by 75 mM K<sup>+</sup>. (D) Activity over time (AoT) images of the primary (red) and secondary (green) responses to nicotinic receptor stimulation (DMPP, 10  $\mu$ M, 20 sec) of the same group of cells as indicated in (C). (E) Ca<sup>2+</sup> responses of 2 varicosities and 2 glial cells (color-coded numbers in C and numbers in D) upon DMPP application. Note the fast upstroke and reverberating activity in neuronal varicosities and the delayed secondary Ca<sup>2+</sup> transients in enteric glial cells. See also **supplementary movie 1**. (F) Gray scale image of a patch of cultured mouse enteric neurons and glia loaded with Fluo4 at rest. (G-H) AoT images of the same patch of cells as in F in which neuronal fibers responding directly (G) and cells displaying a slow Ca<sup>2+</sup> response (H) to electrical field stimulation (2 sec, 20 Hz) are shown. (I) Histogram displaying the distances ( $\mu$ m) from an active neuronal component to cells with (red, n= 135) and without (black, n = 134) secondary responses to nerve stimulation (p<0.05, Fisher's exact). (J) Image of the same cells as in F in which the distance from each pixel to an active neuronal component is color-coded. Scale bars: 50  $\mu$ m (A, F), 20  $\mu$ m (C, D).

**Figure 4. Detection of neuronal versus glial signals in *ex vivo* enteric nervous system preparations.** (A) Gray scale image of a colonic myenteric plexus ganglion loaded with Fluo4 at rest. (B-D) Magnification of the square region marked in A before (B) and after (C, D) electrical stimulation (ETS, 2 sec, 20 Hz) of an interganglionic connective. (E) Activity over time (AoT) image of the region in which pixels responding immediately (red) or with a delay (green) to ETS are false colored. Arrowheads point to enteric glial cells that display a Ca<sup>2+</sup> transient secondary to neuronal stimulation. Dashed line (1) and (2) mark the neuronal

fiber and glia cell used in F and G. **(F)** Fluo4 traces of the regions of interest (color-coded in **B-D**) showing responses upon ETS. **(G)** Magnification of the squared box in **F**. Although the initial increase in the purple and green trace is due to a neuronal fiber (1) crossing the glial cell (2) it is still possible, because of the differences in upstroke speed to distinguish between neuronal and glial cell types  
Scale bars: 50  $\mu\text{m}$  (**A**), 20  $\mu\text{m}$  (**B**, **E**).

**Figure 5. Optogenetic  $\text{Ca}^{2+}$  imaging of enteric neuron-glia crosstalk.** **(A)** Gray scale image of myenteric plexus ganglia dissected from a Wnt1-Cre;R26R-GCaMP3 mouse colon displaying baseline GCaMP3 fluorescence. **(B)** False-colored image of fluorescence response to 75 mM  $\text{K}^+$  depolarization (5 sec) of the same ganglia as shown in **(A)**. **(C-D)** Activity over time (AoT) images of the myenteric ganglion in the frame indicated in **A** in which only pixels responding to electrical stimulation (ETS, 2 sec, 20 Hz) of an interganglionic connective (**C**, red, see also **supplementary movie 2**) or local ATP (10  $\mu\text{M}$ , 20 sec) stimulation (**D**, blue, see also **supplementary movie 3**) are shown. **(E)** GCaMP3 fluorescence image of the same ganglion as in **C** and **D** immunostained for Sox10 (magenta). Arrows indicate enteric neurons displaying a  $\text{Ca}^{2+}$  transient upon electrical stimulation only. Arrowheads point to enteric glial cells responding to ATP stimulation. Asterisk indicates an enteric neuron that responds to both electrical and purinergic stimulation. **(F)** Gray scale images of a patch of cultured myenteric neurons and glia established from a Wnt1-Cre;R26R-GCaMP3 animal, before (0''), during (12'') and after (20'', 40'') stimulation with 75 mM  $\text{K}^+$  (5 sec). **(G)** AoT image of the cells shown in **F** responding immediately (red) or with a delay (green) to 75 mM  $\text{K}^+$ . See also **supplementary movie 4**. **(H)** Recordings of the GCaMP3 responses to 75 mM  $\text{K}^+$  of one neuron and four surrounding enteric glial cells (color-coded numbers in **G**). Neurons typically show an immediate  $\text{Ca}^{2+}$  transient to 75 mM  $\text{K}^+$  while enteric glial cells only respond with a delay, thus indicating neuron-to-glia communication. Note that *post-hoc* examination of cellular identity is redundant because of the genetically-imposed reporter expression.  
Scale bars: 50  $\mu\text{m}$ .

**Figure 6. Interactions between enteric neurons and glia in the human enteric nervous system.** **(A)** Individual confocal images of a human submucosal ganglion obtained from a duodenal biopsy in which enteric neurons are labeled with antibodies for HuCD (green) and NF-200 (red) and enteric glia are immunostained for S100 $\beta$  (blue). **(B)** Maximum projection of the same ganglion as in **A** with orthogonal X (bottom) and Y (right) views. Note how enteric neurons and glial cells are closely packed together within a dense ganglionic capsule. **(C)** Gray scale fluorescence image of a human submucosal ganglion loaded with the  $\text{Ca}^{2+}$  indicator Fluo4. **(D)** Image of the same ganglion as in **C**, immunostained for HuCD (green) and S100 $\beta$  (magenta). **(E)**  $\text{Ca}^{2+}$  responses of a neuronal cell body and two glial cell processes (color-coded numbers in **C** and numbers in **D**) upon 75 mM  $\text{K}^+$  depolarization.  
Scale bars: 25  $\mu\text{m}$  (**A**, **B**), 50  $\mu\text{m}$  (**C**).

Figure 1.TIF

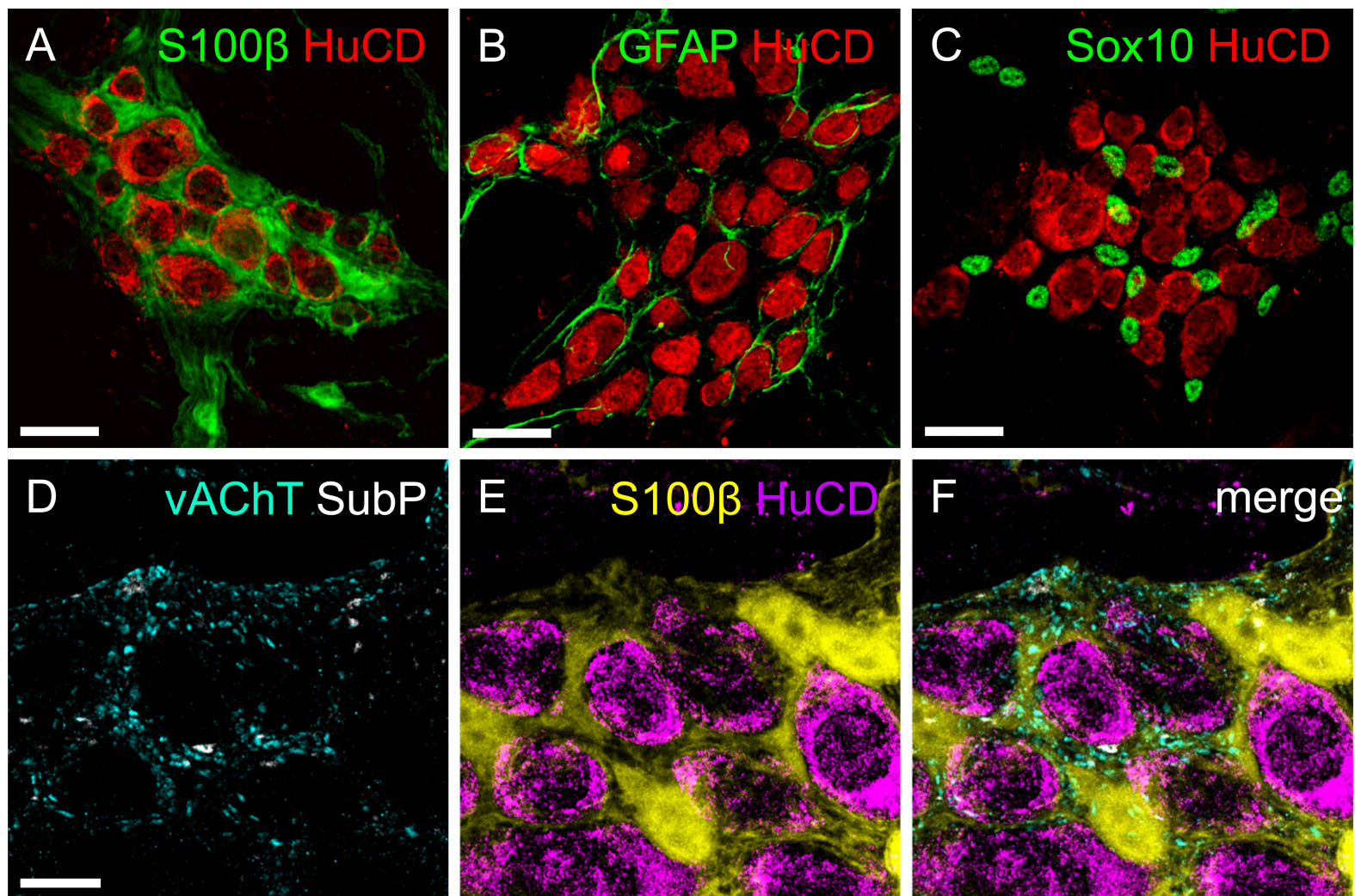




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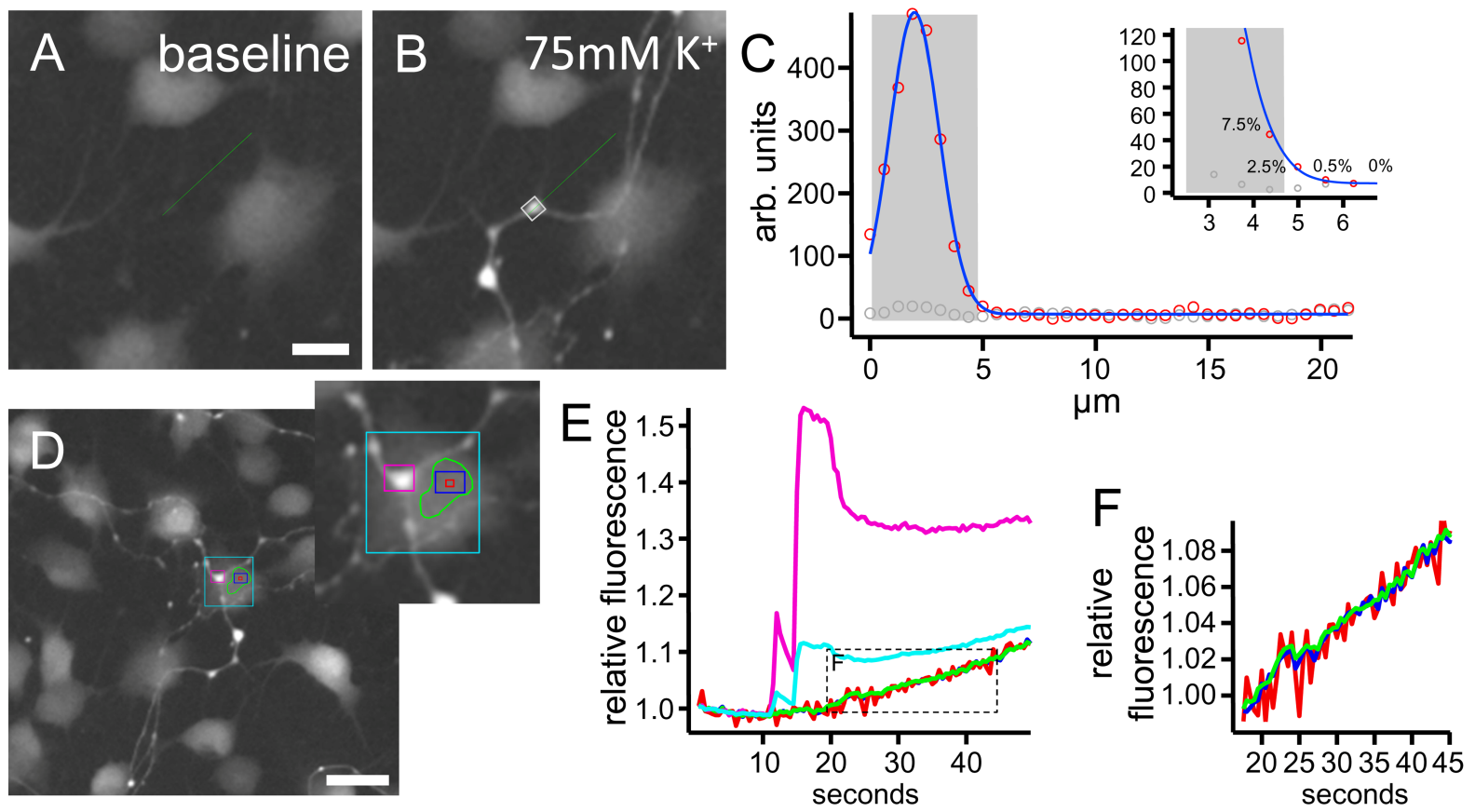




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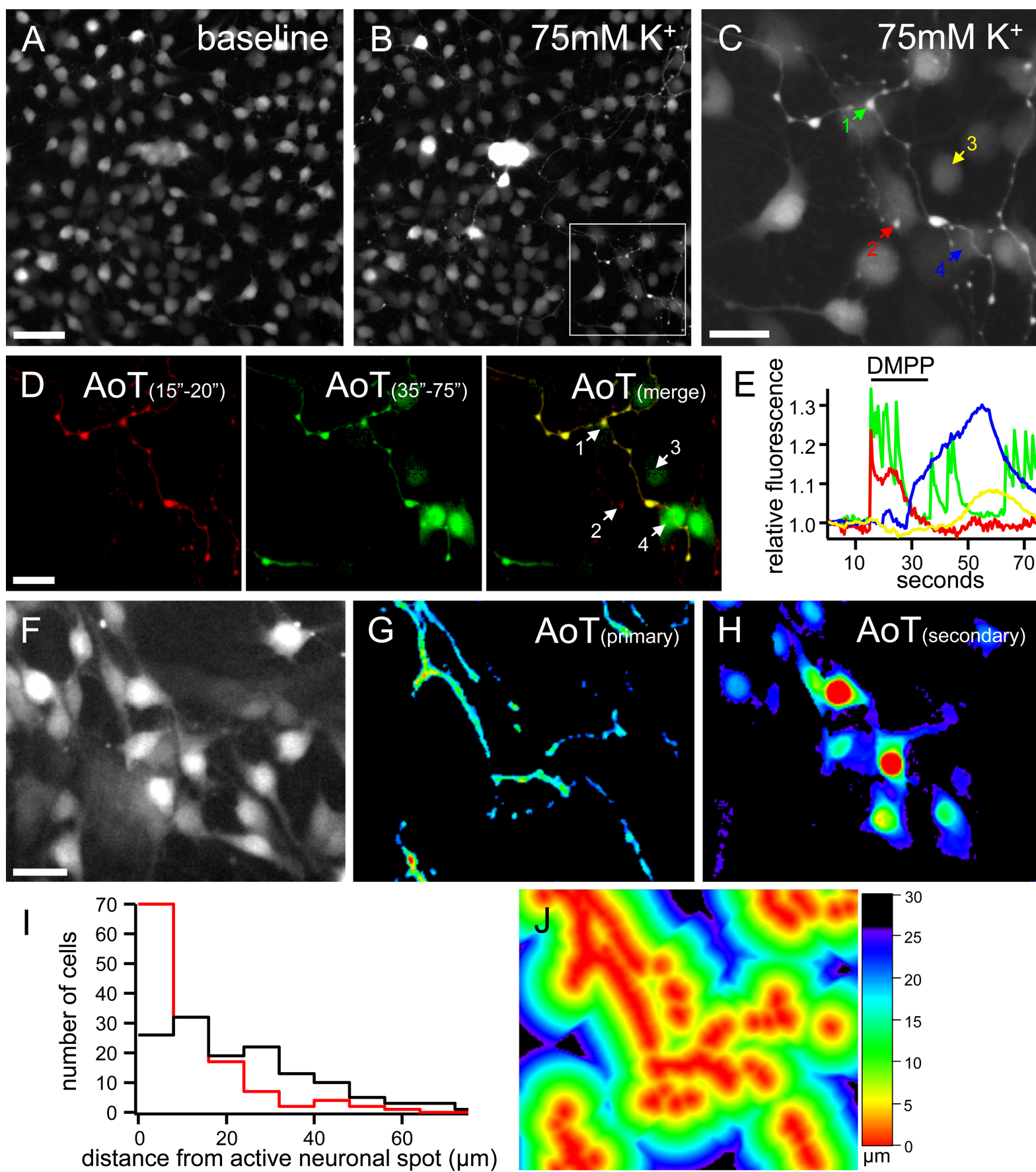


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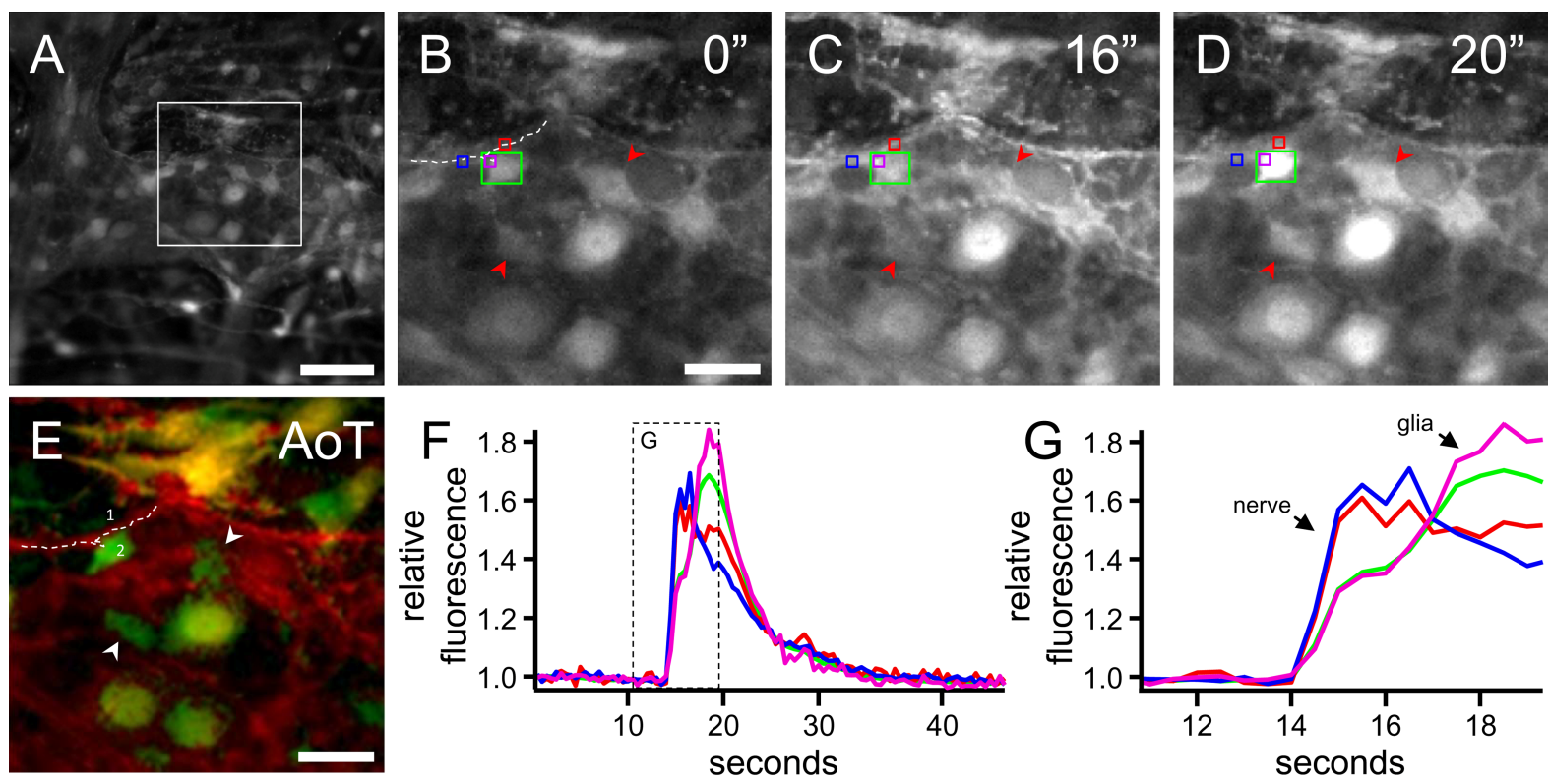




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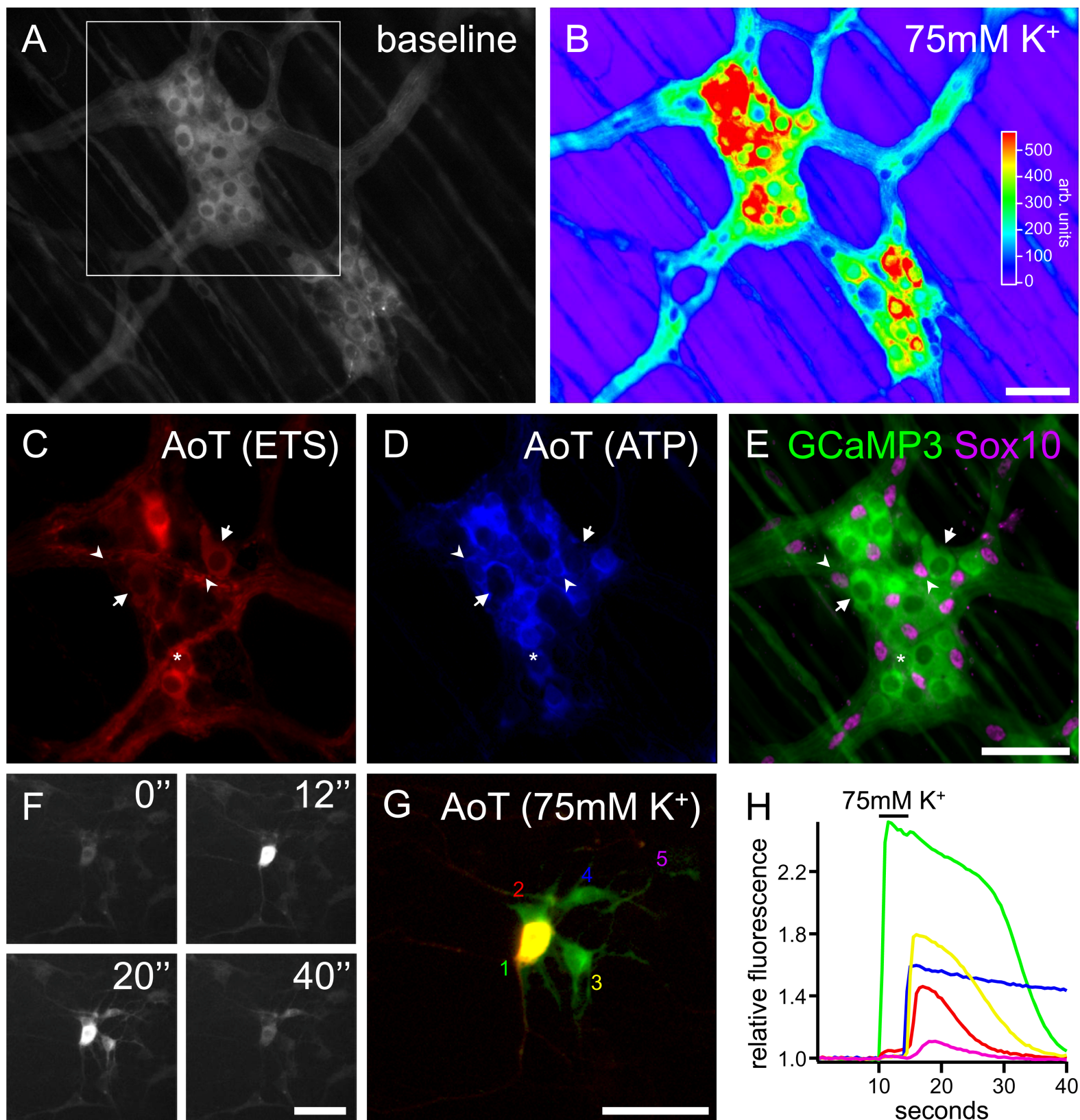


Figure 6.TIF

